# Isolation and Identification of *Mycoplasma mycoides* Subspecies *mycoides* Small Colony Bovine Biotype in Eastern Ethiopia

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## ABSTRACT

An outbreak of contagious bovine pleuropneumonia (CBPP) was investigated in the Somali National Regional State, Eastern Ethiopia, to isolate and identify the causative *Mycoplasma* species. Detailed physical clinical examination was performed on over 700 affected animals to select acutely sick animals for autopsy and bacteriological specimen collection. Postmortem examination and sample collection was performed on 7 recently dead animals. The clinical and pathological findings encountered, and the bacteriological as well as the biochemical tests performed, established the outbreak to be CBPP. Regular mass vaccination of susceptible herds is recommended to control the disease.

## INTRODUCTION

Contagious bovine pleuropneumonia (CBPP) is an economically important, contagious respiratory disease of cattle that affects domestic ruminants of the genus *Bos*, mainly *Bos taurus* and *Bos indicus*. CBPP has become a serious obstacle to livestock development in sub-Saharan Africa and Asia. It is characterized by a morbidity rate of 75%–90%, a mortality rate from 50% to 90%, and a case-fatality rate of 50%.[1]

CBPP is caused by *Mycoplasma mycoides* subspecies *mycoides* small colony bovine biotype of the class Mollicutes.[2] These prokaryotic eubacteria have no cell wall, are able to pass through some Millipore filters, and are hardly visible through an optical microscope.

CBPP has been recognized in Ethiopia for many years, although sufficient information is lacking on the epizootiology of the disease. CBPP was thought to be a problem of lowland pastoral areas, with incursions into the adjacent highlands. Seroprevalence studies conducted during the 1970s revealed CBPP to be present at rates of 0%[3] and 1.78%[4] in the Southern and Eastern Ethiopian lowlands, respectively. A serological survey study carried out in the late 1990s showed the prevalence of CBPP to vary from 10%[5] to 54%[6] in the Western and Northwestern Ethiopian highlands, respectively. The recent outbreaks in and around the nation's capital,[7] Addis Ababa, increases the risk it carries to the dairy sector.

CBPP has now gained a grip in Ethiopia, and is causing subtle economic losses on the agricultural sector and the national economy. It accounts for a loss of over ETB 205.6 million (Ethiopian Birr) per year.[7] All regional states of Ethiopia except the Somali region were classified as either being CBPP endemic or epidemic.[8] Until now there has been no attempt to clarify the extent of CBPP infection in the Somali region of Eastern Ethiopia. The objective of this investigation was therefore to isolate and identify *Mycoplasma mycoides* subspecies *mycoides* small colony bovine variant in the Somali region of eastern Ethiopia.

## MATERIALS AND METHODS

### Study Area, Animals, and Specimens

The present study was conducted in the Jijjiga zone of the Somali National Regional State, east of Addis Ababa. The field investigation encompasses more than 700 African zebu cattle affected by the CBPP outbreak. Detailed physical examinations were carried out in affected animals to select those acutely infected, and necropsy investigations were performed on 7 sacrificed animals.

Specimens of pleuritic fluid, mediastinal lymph nodes, and pneumonic lung tissues were collected for laboratory investigation. Collected samples were kept at –20˚C until inoculation.

### Media used

Modified Hayflick's media as described by the Office International Des Epizooties (OIE)[9] was used for isolation. The medium is composed of *Mycoplasma* broth (18 g), horse serum (200 mL), yeast extract (100 mL), 50% glucose solution (2 mL), 50% sodium pyruvate (8 mL), 2% DNA (1 mL), penicillin (200,000 IU), distilled water (700 mL), and phenol red (0.002 g). The pH was adjusted to 7.8. Solid media was prepared by mixing equal volumes of 2% noble agar and broth medium; 20 mL of the molten medium was spread on 90-mm sterile Petri dishes.

### Inoculation of the medium

The pleural fluid was inoculated directly into the broth media while the tissue samples were homogenized in broth media with sterile mortar and pestle. Two 10-fold dilutions of the homogenate were made and broth cultures inoculated with $10^2$ dilution. The broth was incubated aerobically at 37˚C and checked daily for evidence of growth. The broth culture was harvested at 96 hours with a sterile syringe and passed through a 0.45-µm membrane filter; 0.2 mL of the filtrate was spread on agar plates. The plates were incubated at 37˚C for 120 hours in a humidified chamber. The plates were examined with inverted microscope (32X) for *Mycoplasma* microcolonies. This procedure was repeated three times to obtain pure cultures of the isolate.

### Biological and Biochemical tests

***Sterol dependence test.*** The sterol dependence test was performed in two ways. First, a broth culture of *Mycoplasma* isolate was inoculated into a universal bottle containing growth media without serum. A similar bottle of growth media containing 20% horse serum was inoculated with the same isolate. Both bottles were incubated at 37˚C for 96 hours. Growth was monitored daily based on turbidity and color change. Second, solid media was flooded with 0.01 mL of broth culture of the isolate. Sterile filter-paper discs (6 mm) impregnated with 0.025 mL of digitonin 1.5% weight/volume in ethanol was placed on the center of the agar surface. After allowing the culture to dry in the laminar airflow cabinet for 1 hour, the plate was incubated in a humidified chamber at 37˚C for 3–10 days. The zone of inhibition around the disc was measured from the edges of the disc to determine sensitivity. Inhibition of 5 mm or higher was considered positive.

***Glucose fermentation.*** Two bijoux bottles containing 2.25 mL glucose (0.4%) medium were each inoculated with 0.25 mL of broth culture of the isolate. The bottles were incu-

bated aerobically at 37˚C for 10 days. Fermentation status was checked daily by noting a color change from pink to yellow. The pH of the medium was adjusted to 7.8.

***Arginine hydrolysis.*** Two bijoux bottles containing 2.25 mL of 0.2% weight/volume of arginine medium were inoculated with 0.25 mL of broth culture of the isolate and incubated aerobically at 37˚C for 10 days. The pH of the medium was adjusted with sodium hydroxide to 7.0. The result of the hydrolysis was checked daily by observing a color change to deep purplish-red.

***Urea hydrolysis.*** Ten percent urea was incorporated into the *Mycoplasma* growth media at a final concentration of 1% in the media. Two bijoux bottles with 2.25 mL of the urea medium were inoculated with 0.25 mL of the broth culture of the isolate and incubated at 37˚C for 10 days aerobically. The pH of the medium used was 6.5. Urea breakdown was checked based on observed color change to deep purplish-red.

***Antibiotic sensitivity.*** Antibiotic sensitivity was carried out by metabolic inhibition tests. The concentrations of the antibiotics used were 0.6 mg/L tetracycline, 9 mg/L chloramphenicol, and 200,000 IU penicillin as recommended by Parker and Collier.[10] A total of 0.25 mL of the broth culture of the isolate was inoculated into universal bottles containing growth media with the indicated antibiotics. The bottles were incubated at 37˚C for 10 days. Growth was monitored daily by observing turbidity and color change.

***Growth inhibition.*** A total of 150 µL of the broth culture suspension were placed at one end of the agar plate and the plate tilted so that the inoculum spread evenly over the whole agar plate. The plate was allowed to dry in the laminar airflow cabinet for 30 minutes. A well was made at the center of the agar surface with about 5 mm of metal tubing; 50 µL of monoclonal antibody was added into the well and the plate was left in the cabinet for 2 hours. The plate was then turned upside down and incubated in a humidified chamber at 37˚C for 10 days. Development of a microcolony and zone of inhibition were examined between days 3 and 10 post-inoculation using an inverted microscope (16X). The diameter of the inhibition zone was measured in millimeters from the edges of the well. An inhibition zone of 2 mm or more was considered positive.

## RESULTS

### Clinical and Necropsy Findings

Clinical examinations of infected animals revealed nasal discharge, coughing, labored breathing, disinclination to move, and postures that showed the animal was fighting to get enough oxygen. The profound lesions observed on postmortem showed adhesion of the pleura with the chest wall and the lung, and consolidated lung tissues with characteristic marbling. The pleural cavity was full of copious, yellowish-colored clear fluid. Heavy deposits of fibrin flocculates were encountered.

### Bacteriological Findings

Evidence of the growth of *Mycoplasma* organisms was based on a change in color of the growth medium from pink to yellow. Moderate turbidity with a whitish deposit at the bottom of the culture vessels were additional parameters used to determine *Mycoplasma* growth. Both the tissue samples processed and the pleural fluid cultured were positive for *Mycoplasma* growth after incubation for 72–120 hours in broth culture media. Gram-stained smears from these cultures showed the presence of gram-negative, pleomorphic organisms composed of coccoid, cocco-bacillary, and filamentous organisms. Giemsa-stained preparations from the culture suspensions revealed coccoid, pear-shaped, and filamentous microorganisms. Growth on solid medium was characterized by the presence of microcolonies with a typical nipple-shaped appearance after 7 days of incubation. The colonies were observed under inverted microscope (32X) with transmitted light.

**Table 1.** Summary of Biochemical and Biological Properties Exhibited by *Mycoplasma* Colonies

| | |
|---|---|
| Growth in aerobic conditions | Positive |
| Glucose fermentation | Positive |
| Arginine hydrolysis | Negative |
| Urea breakdown | Negative |
| Sterol dependence | |
| Growth on serum-free media | Negative |
| Digitonin sensitivity | Positive (9 mm)* |
| Ability to pass through 0.45-$\mu$m membrane | Positive |
| Growth inhibition | Positive (> 4 mm)* |
| Antibiotic sensitivity | |
| Penicillin | Negative |
| Chloramphenicol | Positive |
| Tetracycline | Positive |
| Colony morphology | Classical nipple shaped |

*Zone of inhibition.

**Biochemical and Biological Properties**

Table 1 shows a summary of the biochemical and biological properties of the *Mycoplasma* microcolonies. Positive results were seen for growth in aerobic conditions, glucose fermentation, digitonin sensitivity, ability to pass through a 0.45-µm membrane, growth inhibition, and sensitivity to chloramphenicol and tetracycline. Negative results were seen for arginine hydrolysis, urea breakdown, growth on serum-free media, and sensitivity to pencillin.

## DISCUSSION

The clinical and pathological findings observed in this investigation are consistent with the results of previous studies.[1,7,11–13] Similarly, the bacteriological, biochemical, and biological properties of the isolate reported in the present study are in agreement with reports in the literature.[10,11,14,15] The goal of the antibiotic sensitivity test was not to determine the antibiotic susceptibility pattern of the organisms; rather, it was used to aid in the identification of *Mycoplasma* isolates, as has been described by Fallon and Whittlestone.[16] It is well established that *Mycoplasma* is resistant to beta-lactams but are sensitive to antibiotics targeted at inhibition of protein synthesis.[10] The results of the sensitivity test were in agreement with those of other investigators.[10,16]

We confirm that the outbreak studied was CBPP, clearly demonstrating that this disease is still active in Ethiopia and providing the first confirmed diagnosis of CBPP in the Somali regional state. The region is now considered one of the CBPP-epidemic areas.

We have isolated a virulent field strain of the causative *Mycoplasma,* which can be used as a challenge preparation to assess the impact of antibiotic therapy on the epidemiology of CBPP as well as the efficacy of CBPP vaccination, which has not been possible in Ethiopia due to lack of a challenge strain. It has been suggested that the current vaccines are not efficacious against virulent field strains of the causative *Mycoplasma* in Eastern and Southern African regions.[13] The current isolate is, therefore, a good candidate to be used to create an efficacious CBPP vaccine. Once this is accomplished, mass immunization of the susceptible herd is recommended to control the disease.

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